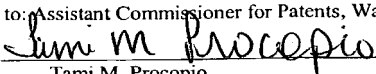


JCO2 Rec'd PCT/PTO 03 APR 2002

FORM PTO-1355 OFFICE (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK		ATTORNEY'S DOCKET NUMBER  246152016800	
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371</b>				U.S. APPLICATION NO. (If known, see 37 CFR 1.5)  <b>10/089879</b>	
INTERNATIONAL APPLICATION NO.  PCT/EP00/09872		INTERNATIONAL FILING DATE  3/10/2000		PRIORITY DATE CLAIMED  4/10/1999	
TITLE OF INVENTION  <b>DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS</b>					
APPLICANT(S) FOR DO/EO/US  <b>Pieter Cornelis LANGEVELD and Jacobus STARK</b>					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> An English language translation of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made, however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).					
<b>Items 11. to 16. below concern document(s) or information included:</b>					
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input type="checkbox"/> Other items or information: *, return receipt postcard.					
<b>CERTIFICATE OF MAILING BY "EXPRESS MAIL"</b>  Express Mail Label No EV 042193580 US      Date of Deposit: April 3, 2002  I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.  <div style="text-align: center;">         Tami M. Procopio     </div>					

U.S. APPLICATION NO (if known, see 37 CFR 1.5) * <div style="font-size: 1.5em; font-weight: bold;">10/089879</div>		INTERNATIONAL APPLICATION NO PCT/EP00/09872		ATTORNEY'S DOCKET NUMBER: 246152016800	
21. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$1,000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provision of PCT Article 33(1)-(4) .....\$690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) .....\$100.00					<b>CALCULATIONS</b> PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$890.00	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$*	
Total claims	9- 20 =	0	x \$18.00	\$	
Independent claims	3 - 3 =	0	x \$80.00	\$*	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$*	
TOTAL OF ABOVE CALCULATIONS =				\$1020.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				\$	
SUBTOTAL =				\$1020.00	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$*
TOTAL NATIONAL FEE =				\$*	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00 per property</b>				+	\$*
TOTAL FEES ENCLOSED =				\$1020.00	
				Amount to be refunded:	
				charged:	\$1020.00
a. <input type="checkbox"/> A check in the amount of \$* to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my <b>Deposit Account No. 03-1952</b> in the amount of \$1020.00 to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to <b>Deposit Account No. 03-1952</b> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">           SEND ALL CORRESPONDENCE TO:             Kate H. Murashige            Morrison &amp; Foerster LLP            3811 Valley Centre Drive            Suite 500            San Diego, California 92130-2332         </div> <div style="width: 45%; text-align: center;"> <div style="font-size: 1.2em; font-family: cursive;">Kate H. Murashige</div> <div style="border-top: 1px solid black; width: 100%; margin: 2px 0;"></div>           SIGNATURE             Kate H. Murashige            Registration No. 29,959         </div> </div>					

10/089879

JC13 Rec'd PCT/PTO 03 APR 2002

PATENT

Docket No. 246152016800

Client Reference 02970USWO EM

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*Tami M Procopio*  
Tami M Procopio

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In the application of:

Pieter Cornelis LANGEVELD  
and Jacobus Stark

Serial No.: To be assigned

Filing Date: Even date herewith

For: DETECTION OF ANTIMICROBIAL  
RESIDUES IN EGGS

Examiner: To be assigned

Group Art Unit: To be assigned

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Prior to examination of the above-referenced application, please amend the claims as follows:

Enclosed is the following Exhibit A: Marked-up Version of Amendments to the Claims.

## AMENDMENT

### **In the Claims:**

#### **Please replace the presently pending claims with the following claims:**

1. (Amended) A process for determining the presence or absence of an antimicrobial residue in a sample of an egg which process comprises:
  - (i) contacting the sample with a test suitable for determining the presence or absence of an antimicrobial residue in the sample;
  - (ii) heating the contacted sample and test for a sufficient time interval to inactivating any natural inhibiting compound present in the sample; and
  - (iii) incubating the contacted sample and test, to determine whether microbial growth occurs,whereby the absence of microbial growth indicates the presence of at least one antimicrobial residue.
2. (Amended) A process according to claim 1, wherein said heating is to a temperature of from 70°C to 100°C.
3. (Amended) A process according to claim 2, wherein said heating is to a temperature of from 75°C to 85°C.
4. (Amended) A process according to claim 1, wherein said heating is from 2 to 20 minutes.
5. (Amended) A process according to claim 4, wherein said heating is from 10 to 15 minutes.
6. A process according to claim 1 wherein the test comprises a test organism, nutrients and one or more indicators present in an agar medium.

Please cancel claim 7.

8. (Amended) A test kit for determining the presence or absence of an antimicrobial residue in a sample of an egg, which test kit comprises:

(i) a test suitable for determining the presence or absence of an antimicrobial residue in a sample; and

(ii) a sample,

wherein any lysozyme present in the sample has been inactivated.

Please cancel claims 9-10.

11. (Amended) A computer program in combination with a computer, whereby the program causes the computer to operate in such a way that the computer controls the temperature of an incubator, whereby after starting the program, the temperature will be set at a temperature for a selected inactivation time interval whereby lysozyme will be inactivated in an egg sample, whereafter the temperature is set at a temperature whereby incubation of the test sample takes place during a selected incubation time interval.

Please add the following claims:

12. (New) The process of claim 1, wherein said natural inhibiting compound is lysozyme.

**REMARKS**

The claims have been amended to clarify the wording, conform to U.S. practice, and eliminate multiple dependencies. No new matter has been added and entry of the amendment is respectfully requested.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket No. 246152016800.

Respectfully submitted,

Dated: April 3, 2002

By: Kate H. Murashige  
Kate H. Murashige  
Registration No. 29,959

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Telephone: (858) 720-5112  
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**EXHIBIT A. - VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**In the Claims:**

1. (Amended) A process for determining the presence or absence of an antimicrobial residue in a sample of an egg which process comprises:
  - (i) contacting the sample with a test suitable for determining the presence or absence of an antimicrobial residue in the sample;
  - (ii) heating the contacted sample and test for a sufficient time interval to [inactivate a] inactivating any natural inhibiting compound[, for example lysozyme,] present in the sample;and
  - (iii) incubating the contacted sample and test, to determine whether microbial growth occurs,  
whereby the absence of microbial growth indicates the presence of at least one antimicrobial residue.
2. (Amended) A process according to claim 1, wherein [the contacted sample and test are heated] said heating is to a temperature of from 70°C to 100°C.
3. (Amended) A process according to claim 2, wherein [the contacted sample and test are heated] said heating is to a temperature of from 75°C to 85°C.
4. (Amended) A process according to [any one of claims 1 to 3] claim 1, wherein [the sample and test are heated for] said heating is from 2 to 20 minutes.
5. (Amended) A process according to claim 4, wherein [the sample and test are heated] said heating is from 10 to 15 minutes.
8. (Amended) A test kit for determining the presence or absence of an antimicrobial residue in a sample of an egg, which test kit comprises:

(i) a test suitable for determining the presence or absence of an antimicrobial residue in a sample; and

(ii) [the] a sample,

wherein any lysozyme present in the sample has been inactivated.

11. (Amended) A [computer] computer program in combination with a [computer] computer, whereby the program causes the [computer] computer to operate in such a way that the [computer] computer controls the [temperaure] temperature of an incubator, whereby after starting the program, the temperature will be set at a temperature for a selected inactivation time interval whereby lysozyme will be inactivated in an egg sample, whereafter the temperature is set at a temperature whereby incubation of the test sample takes place during a selected incubation time interval.



## DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS

Field of the invention

5 The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in eggs.

Background of the invention

10 The presence of antimicrobial residues in food and feed is a growing concern among the consumers due to health-related problems and the increase of drug resistant bacteria. Antibiotics are not only applied as medication, but certainly in case of poultry also widely used as antimicrobial growth promoting substances. It is well known that concentrations of antimicrobial residues in eggs may be high. In most countries, such as the countries of the European Union, Canada and the  
15 United States, Maximum Residue Levels (MRL) are regulated by legislation.

Test methods to detect antimicrobial residues in body liquids such as milk or urine such as microbial inhibition tests (e.g. agar diffusion tests) or methods making use of selective binders (e.g. antibodies or tracers) are well known. Examples of microbiological test methods have been described in GB-A-1467439, EP 0005891,  
20 DE 3613794, CA 2056581, EP 0285792 and US 5494805. These documents all deal with ready to use tests that make use of a test organism. The test organism is mostly imbedded in an agar medium, which may contain an indicator, a buffer solution, nutrients and substances to change the sensitivity for certain antimicrobial compounds in a positive or negative way.

25 Examples of suitable test organisms are strains of *Bacillus*, *Streptococcus* or *E.coli*. In general, the principle of these tests is that when antibacterial compounds are present in a sample at a concentration sufficient to inhibit the growth of the test organism the color of an acid/base or redox indicator will stay the same. However when no inhibition occurs, growth of the test organism is accompanied by the  
30 formation of acid or reduced metabolites leading to a change in the colour of the indicator.

These test methods are suitable for the detection of antimicrobial residues in body liquids. However up to now detection of antimicrobial residues in eggs was not possible due to the presence of antimicrobial substances, such as lysozyme, which  
35 are naturally present in high concentrations in eggs. These inhibiting substances

show inhibitory activity against the test micro-organism leading to false positive results.

In case of e.g. a milk or urine sample inhibiting substances such as lysozyme or lactoferrin can be inactivated by heating the sample, e.g. at 80°C for 10 minutes (Vermunt et. al., Netherlands Milk and Dairy Journal 47: (1) 31 – 40 (1993)), or by using well known dialysis methods (van Wall, Archiv für Lebensmittelhygiene 29: (6) 235 (1978)). After this pre-treatment the liquid sample can be used for further testing simply by following the procedures of the test. In case of a Delvotest® type of test (EP 0005891) the liquid sample can be added directly to the test, after which the test is incubated.

However heating of an egg sample at temperatures sufficient to inactivate inhibiting substances of the egg, such as lysozyme, always leads to coagulation of the sample. It was believed that such samples are not suitable for further processing anymore.

Up to now after heating at a temperature sufficient to inactivate antimicrobial substances other than antimicrobial residues, the antimicrobial residues to be detected have to be extracted from the coagulated egg sample. These extraction methods not only cost a lot of time and extra handling but even worse always lead to loss of at least part of the antimicrobial residues, if present in the sample (Inglis et. al., Journal for the Association of Official Analytical Chemists 61: (5) 1098 – 1102 (1978); Katz et. al., Journal of the Association of Official Analytical Chemists 61: (5) 1103 – 1106 (1978); Janetschke et. al., Monatshefte für Veterinaermedizin 34: (21) 824 – 826 (1979); Steiner, Monatshefte für Veterinarmedizin 45: (11) 382 – 386 (1990)). This may lead to false negative results and therefore antibiotics in consumer eggs, which of course is unacceptable from a health point of view. Moreover laboratories executing studies concerning the presence or absence of antimicrobial residues in foods are limited by the time available to execute these studies. With the present time consuming methods only a very limited amount of egg samples can be examined. Further, these assays can only be executed in well-equipped laboratories and by well-educated persons, which is also a limiting factor.

It can be concluded that up to now no suitable test method for the detection of antimicrobial residues in egg samples is available. The present methods are unreliable, time consuming and may lead to both false positive and false negative results, which in turn leads to unacceptable amounts of antibiotics in the food chain and to economic losses.

### Detailed description of the invention

The present invention provides a reliable and simple to carry out, one-step test for the detection of antimicrobial residues in eggs.

5 Unexpectedly it has been found that when an egg sample is added to a test suitable for detecting antimicrobial residues and then is incubated for a sufficient time at a sufficient temperature to inactivate natural inhibiting compounds of the egg, the test can be incubated directly after heating to determine the presence or absence of antimicrobial residues.

10 It has been surprisingly found that antimicrobial residues diffuse directly from the coagulated egg sample into the test system. Thus additional extraction methods to obtain the antimicrobial residues from the coagulated egg sample are not required.

According to the invention there is thus provided a process for determining  
15 the presence of an antimicrobial residue in an egg which process comprises:

- (i) contacting the sample with a test suitable for determining the presence or absence of an antimicrobial residue in the sample;
- (ii) heating the contacted sample and test for a sufficient time interval to inactivate a natural inhibiting compound e.g. lysozyme present in the sample;
- 20 and
- (iii) incubating the contacted sample and test.

By natural inhibiting compounds are meant compounds which may disturb the test and which are naturally present in the sample such as naturally inhibiting compounds, for example lysozyme. Thus by disturbing or inhibiting is meant the  
25 behaviour of the compound on parts of the test, for example the test micro organism. The invention also provides test kit for determining the presence or absence of an antimicrobial residue in a sample of an egg, which test kit comprises:

- (i) a test suitable for determining the presence or absence of an antimicrobial residue in a sample; and
  - 30 (ii) the sample,
- wherein natural inhibiting compounds e.g. lysozyme present in the sample have been inactivated.

The exact time/temperature requirements depend on e.g. the condition of the sample (e.g. the starting temperature, the volume of the sample, whole egg, egg  
35 white or egg yolk); the type of test (e.g. microbial inhibition tests or assays based on selective binders (e.g. antibodies or tracers)); or the microorganism used in the test

(e.g. thermophilic or non-thermophilic *Bacillus* or *Streptomyces* species). Of course it should be taken care of that the heat treatment will not inactivate the antimicrobial residues to be detected. The heat treatment can be executed using any method known in the art, e.g. by heating in a water bath or by using an incubator as described below.

For example the test can be performed in the following way:

1. A sample of the egg is obtained by making a hole in the egg of e.g. approximately 1-2 square cm, prick the egg-yolk, place the egg with the hole down on a bottle, after the egg is empty the bottle is closed and the sample is homogenized by shaking. Alternatively of course any other method known in the art to obtain a sample of the total egg, egg white or egg yolk can be used;
2. Add a sufficient amount of the egg sample to be tested to a test using well known methods;
3. Heat the test, e.g. for approximately 10 minutes at 80° C, to inactivate the natural inhibiting substances (e.g. lysozyme), to coagulate the egg sample;
4. Incubate the test following the standard procedures of the test and read the result.

Any test suitable for determining the presence or absence of antimicrobial residues may be used in a process of test kits of the invention. Examples are described in GB-A-1467439, EP-0005891, DE-3613794, CA-2056581, EP-028579 and US 5,494,805 which are incorporated herein by reference. Suitable tests are those in which selected sensitive microorganisms are used, e.g. microbial agar diffusion tests, or tests based on selective binding of the compound to be detected. Selective binding can be achieved using the well-known antibody technology or by using specific tracers. An example of a specific tracer is the penicillin binding protein, which is used in e.g. the Delvo-X-Press® for detecting beta-lactams.

Examples of suitable microbial agar diffusion tests are tests in which species of *Bacillus*, *Streptococcus* or *E. coli* are used. Preferably thermophilic species, e.g. *Bacillus stearothermophilus* and *Streptococcus thermophilus* are used. Examples of preferred strains are *Bacillus stearothermophilus* var. *calidolactis* C953 (deposited with the Laboratory of Microbiology of the Technical University of Delft under the accession number LMD 74.1 in 1974 and with the Centraal Bureau voor Schimmelcultures (CBS), Baarn under the accession number CBS 760.83 in 1983

were the strain is available to the public) and *Streptococcus thermophilus* T101 (DSM 4022, deposited on March 3, 1987). Both strains are very sensitive to antimicrobial compounds, especially chemotherapeutics such as sulfa compounds and antibiotics such as penicillins and tetracyclines. *E.coli* strains or other suitable gram-negative bacteria can be used for the detection of e.g. quinolones.

*Bacillus stearothermophilus* var. *calidolactis* C953 and *Streptococcus thermophilus* T101 are fast growing and have the advantage that they are thermophilic. For example the optimum growth temperature of said *Bacillus* strain is from 50° to 70°C. The test organism is therefore very suitable for a test according to the invention as it is not killed by heating to inactivate the natural inhibiting compounds which may be present in the egg sample.

When the test organism is a *Bacillus* strain, it is preferably incorporated into the agar medium in the form of a spore suspension which may be prepared and incorporated into the agar medium prior to solidification by known methods (see for example GB-A-1467439). When the test organism is a *Streptococcus* strain, the bacteria are preferably incorporated into the agar medium in the form of bacterial cells which may be prepared according to known methods (see for example EP 0285792). The concentration of the test organism in the agar medium is preferably between  $10^5$  and  $10^{10}$  colony forming units per ml of agar medium.

Suitable nutrients to enable multiplication of the test organism in the absence of antimicrobial residues are for example assimilable carbon sources (e.g. lactose, glucose or dextrose), assimilable nitrogen sources (e.g. peptone) and sources of growth factors, vitamins and minerals (e.g. yeast extract).

The growth of the test microorganism can be detected using well known methods, preferably by colour change of the agar medium of the test sample. Typically a colour indicator, preferably an acid-base or a redox indicator, is used. Examples of suitable acid-base indicators include bromocresol purple and phenol red. Examples of suitable redox indicators include brilliant black, methylene blue, toluidine blue and Nile blue. Also combinations of two or more indicators can be used.

Optionally the sensitivity of the test may be altered by adding certain substances, by changing the test conditions such as pH or concentration of buffering substances or agar or by varying the ratio of the volumes of agar and egg sample. Examples of substances that may be added to the test system to change sensitivity are nucleosides such as adenosine, or antifolates such as trimethoprim, ormethoprim or tetroxoprim, which improve the sensitivity of the test organism to

sulfa compounds. Salts of oxalic acid or hydrofluoric acid may be added to improve the sensitivity to tetracyclines. Cysteine may be added to diminish the sensitivity to penicillins.

5 The amount of egg sample (whole egg, egg white or egg yolk of any species, preferably poultry) to be added to the test depends on the test system. For microbial diffusion tests typically from 0.01 to 1.0 ml, preferably from 0.05 to 0.5 ml is added to the test using well-known methods. After addition of the egg sample the test is heated to inactivate the natural antimicrobial compounds present in the sample, e.g. lysozyme, of the egg sample. Preferably the test is heated for from 2 to 20 minutes  
10 at from 70°C to 100°C, more preferably the test is heated for from 10 to 15 minutes at from 75°C to 85°C or for from 2 to 6 minutes at about 100°C. Any other time / temperature treatment, which is sufficient to inactivate the natural inhibiting compounds of the egg without inactivating the antimicrobial residues to be detected, may be used.

15 After the heat treatment the test is incubated following the instructions of the test manufacturer. The incubation time of the test is dependent on the circumstances. In case of an agar diffusion tests using *Bacillus stearothermophilus* the test is incubated in a water bath or block heater at from 60°C to 70°C, preferably at from 62°C to 65°C. Typically, results may be obtained after 1.5 to 4 hours,  
20 preferably from 2.5 to 3.5 hours. In case of tests using selective binders, such as antibodies or tracers, the results may be obtained within about 30 minutes.

Conventional microbial inhibition tests suitable for use in the present invention include the commercial products, Delvotest®, Premi®test and BR-test® (obtainable from DSM N.V. Holland) the ADM Copan®test (Copan, Italy) and the  
25 Charm®AIM test (Charm, USA). Inactivation of the natural inhibiting compounds present in the egg sample, e.g. lysozyme, is preferably achieved by heating for example for from 5 to 15 minutes at for example from 75° C to 85°C. Alternatively any other temperature / time treatment, which is sufficient to obtain said effects, can be used.

30 In a further aspect, the invention provides a test kit for carrying out the method of the invention. This test kit contain the test and is suitable to execute the method of the invention: add the egg sample, heat to inactivate the natural inhibiting compounds of the sample, incubate the test and read the results.

Examples of kits useful for the purpose of the invention are transparent  
35 tubes, single or in a set, or combined to a block of translucent material provided with

a number of holes shaped therein (incubator). The test kit may contain solidified agar medium which may be optionally buffered; a test organism (e.g. a strain of *Bacillus* or *Streptococcus*) at sufficient colony forming units; nutrients for growth of said organism; an indicator (e.g. an acid-base or redox indicator); optionally  
5 substances to change the sensitivity for certain antimicrobial compounds in a positive or negative way. All ingredients may optionally be added to the test as a separate source, for example as a tablet or paper disc.

The test kits preferably have determined sizes. This is because of the reliability of the test. In case of a test based on agar diffusion technology, preferably  
10 tubes are used. The test unit will preferably be high enough to contain an amount of agar medium and a sample corresponding to preferably a height of from 3 to 30 mm, more preferably from 5 to 15 mm. The internal cross-sectional dimension of the test units is preferably from 1 to 30 mm, more preferably from 5 to 15 mm. The test units are preferably closed air tight during storage in which conditions they may be stored  
15 for at least several months. Of course any other test unit suitable for executing the method of the invention is included in this invention.

The volume of the agar medium in the test unit is determined by the height of the test unit, the internal cross-sectional dimension of the test unit and the percentage of the volume of the test unit, which is filled with the agar medium. The  
20 volume of the agar medium is preferably from 10 µl to 5 ml, more preferably from 100 µl to 1 ml.

Incubators suitable to execute the heat treatments as described in this invention can be constructed in such a way that after placing the test units in the incubator, heat and incubation treatments as described above can be done. The first  
25 heat treatment to inactivate the inhibiting compounds and optionally to form solid matrix and / or to activate the spores is executed at a higher temperature, after which the incubation of the test may continue at a lower temperature. Optionally after the incubation of the test the incubator can cool down to a temperature sufficient to stop the test.

30 An example of such an incubator is a block heater in which test units (e.g. ampoules) can be placed. For example in case of a conventional microbial agar diffusion test using a *Bacillus stearothermophilus* strain the incubator / block heater may contain a number of holes suitable for placing the test ampoules or test plates (e.g. Delvotest® or Premi®Test) therein. After placing the ampoules or plates the  
35 incubator heats the test to a temperature of e.g. from 75°C to 85°C for e.g. from 10

to 20 minutes after which the incubator turns to a lower temperature of from 62°C to 65°C for from 1.5 to 4 hours (incubation of the test). Of course the exact time / temperature intervals depend on many factors and will differ per type of test. This invention includes all incubators capable to execute a pre-incubation at a certain temperature for a certain period of time directly followed by an incubation at a lower temperature for a certain period of time. Optionally after the incubation of the test the incubator can cool down to a temperature sufficient to stop the test.

The process described in this invention is very simple to carry out, so that persons who perform the test do not have to be specially educated or trained.

All documents mentioned in this application are herein incorporated by reference to the same extent as if each individual application or patent was specifically and individually indicated to be incorporated by reference.

### Example 1

#### Preparation of a whole egg sample

To obtain an egg sample for examination on the presence or absence of antimicrobial residues a hole of approximately 1-2 cm<sup>2</sup> was made in the egg, the egg yolk was pricked and the egg was placed with the hole down on a bottle allowing the egg white and egg yolk to drip into the bottle. After the egg was emptied, the bottle was closed and the sample was homogenized by shaking.

### Example 2

#### Inactivation of natural inhibiting compounds of the egg and examining the samples on Delvotest®

Samples of 5 eggs (duplicate), which did not contain antimicrobial residues, were obtained according to the method described in Example 1. To inactivate the natural inhibiting compounds present in the egg sample, 100 µl of each of the 5 samples was added on Delvotest® ampoules. The test was produced according to the methods described in EP 0005891 with the nutrients present in the agar. After heating for 10 minutes at 80°C in a waterbath, the ampoules were immediately placed in a waterbath at 64°C and incubated following the instructions of the producer. After 140 minutes the colour of all tests turned from purple to yellow, indicating that no antimicrobial residues were present.



Control samples were not heated at 80°C for 10 minutes, but directly placed on the ampoule. These tests remained purple for at least 4 hours.

These results clearly demonstrate that natural inhibiting compounds in the egg sample inhibited the test leading to false-positive results. When the sample was heated as described above, the activity of the natural inhibiting compounds was eliminated and no false-positive results were observed anymore.

### Example 3

#### Determination of the sensitivity of the Delvotest® according to the method described in this invention using spiked samples

Egg samples were obtained according to the method described in Example 1. The samples were spiked by adding Penicillin G (0 and 4 ppb) or Sulphadiazine (0 and 100 ppb). The egg samples were added to Delvotest® ampoules (see Example 2) according to the method described in this invention: heated for 10 minutes at 80°C, and then immediately placed in a waterbath at 64°C and incubated following the instructions of the manufacturer. The results were read as soon as the colour turned to yellow (after 140 minutes). The samples containing no Penicillin G (0 ppb) or Sulphadiazine (0 ppb) were negative, while the samples spiked with 4 ppb Penicillin G and 100 ppb sulphadiazine remained purple (positive).

These results clearly demonstrate that the method described in this invention is suitable for detecting antimicrobial residues in egg samples.

**CLAIMS**

1. A process for determining the presence or absence of an antimicrobial residue in a sample of an egg which process comprises:

5 (i) contacting the sample with a test suitable for determining the presence or absence of an antimicrobial residue in the sample;

(ii) heating the contacted sample and test for a sufficient time interval to inactivate a natural inhibiting compound, for example lysozyme, present in the sample; and

10 (iii) incubating the contacted sample and test.

2. A process according to claim 1, wherein the contacted sample and test are heated to a temperature of from 70°C to 100°C.

15 3. A process according to claim 2, wherein the contacted sample and test are heated to a temperature of from 75°C to 85°C.

4. A process according to any one of claims 1 to 3, wherein the sample and test are heated for from 2 to 20 minutes.

20 5. A process according to claim 4, wherein the sample and test are heated from 10 to 15 minutes.

25 6. A process according to claim 1 wherein the test comprises a test organism, nutrients and one or more indicators present in an agar medium.

7. A process according to claim 6 whereby the degree of growth or inhibition of growth of the test organism is determined indicating the absence or presence of the antimicrobial residue.

30 8. A test kit for determining the presence or absence of an antimicrobial residue in a sample of an egg, which test kit comprises:

(i) a test suitable for determining the presence or absence of an antimicrobial residue in a sample; and

35 (ii) the sample,

wherein lysozyme present in the sample has been inactivated.

9. Use of an antimicrobial inhibition test to test an egg sample on the presence of an antimicrobial residue.

5 10. Use of an incubator for an antimicrobial residue test to inactivate lysozyme in an egg sample.

10 11. A computer program in combination with a computer, whereby the program causes the computer to operate in such a way that the computer controls the temperaure of an incubator, whereby after starting the program, the temperature will be set at a temperature for a selected inactivation time interval whereby lysozyme will be inactivated in an egg sample, whereafter the temperature is set at a temperature whereby incubation of the test sample takes place during a selected incubation time interval.

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number  
**WO 01/25795 A2**

- (51) International Patent Classification<sup>7</sup>: G01N 33/94
- (21) International Application Number: PCT/EP00/09872
- (22) International Filing Date: 3 October 2000 (03.10.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
99203264.9 4 October 1999 (04.10.1999) EP
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- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— Without international search report and to be republished upon receipt of that report.

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS

(57) Abstract: The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in eggs. A one step test method is described in which residues of antimicrobial compounds such as antibiotics are detected while inhibiting compounds naturally present in samples obtained from eggs, which may interfere with the test, are inactivated.

WO 01/25795 A2

#5

PATENT \_\_\_\_\_  
Docket No. 24615 \_\_\_\_\_  
Client Ref. 02970USWO \_\_\_\_\_

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AS A BELOW-NAMED INVENTOR, I HEREBY DECLARE THAT:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and \*[sole/joint] inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: THE DETECTION OF ANTIMICROBIAL RESIDUES IN EGGS, the specification of which is attached hereto unless the following box is checked:

☒ was filed on **03 October 2000** as PCT International Application No. **PCT/EP00/09872**

I HEREBY STATE THAT I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE.

I acknowledge the duty to disclose information which is material to the patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

Application No.	Country	Date of Filing (day/month/year)	Priority Claimed?
99203264.9	EP/NL	04 October 1999	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Serial No.	Filing Date
*	

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Application Serial No.	Filing Date	Status
PCT/EP00/09872	03 October 2000	<input type="checkbox"/> Patented <input checked="" type="checkbox"/> Pending <input type="checkbox"/> Abandoned

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